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14. ABSTRACT We have completed proposed research of the Second Year Task (iii) and the part of Task (iv) by studying: (a) Foci Formation, (b) Anchorage-independent cell growth, and (c) tumor spheroid formation using new 3D HuBiogel bioassay whether estrogen-induced conversion of normal cells to transformed cells is inhibited by treatment with antioxidants, over expression of MnSOD, catalase, PrxIII, Trx2; or mtTFA silencing. Normal breast epithelial cells respond to E2 in terms of producing ROS very similar to breast cancer cells. E2 treatment to MCF-10A cells increased the formation of ROS. Over expression of catalase or silencing of mtTFA prevented E2-induced anchorage-independent growth of MCF-10A cells. We observed similar results using 3-D culture of transformed cells. These results support ROS functioning as signal molecules in E2-induced cell transformation. These findings suggest that, in addition to the receptor activity of E2, E2-generated ROS may promote susceptibility to malignant transformation. Thus our results suggest 1) a new paradigm that estrogen-induced oxidants control cell transformation and invasiveness of transformed cells, and 2) provide the basis for the discovery of novel antioxidant-based drugs or antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer.				
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Annual Report –Second Year

Introduction: Recently, we reported that mitochondria are significant targets of estrogen (Felty and Roy, 2005). There is considerable evidence, both experimental and epidemiologic, that estrogens play a role in carcinogenesis; however, these effects cannot be fully accounted for by the mitogenic effects induced by estrogen stimulation of their receptors (ER) (Roy et al, 2004). Both nongenomic estrogen-induced reactive oxygen and nitrogen species (RO/NS) and direct transcriptional ER effects are required to promote DNA synthesis. In this application, we have proposed to investigate the role of estrogen-induced mitochondrial (mt) oxidant signaling pathways in the *in vivo* progression of breast cancer as a new line of research that may lead to the discovery of novel antioxidant-based drugs or new antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer. We proposed to examine an untested and highly innovative concept in estrogen-induced carcinogenesis research, *i.e.*, estrogen-induced mitochondrial oxidants are involved in the promotion/progression of breast cancer through modulating signaling that controls the early G1 stage of the cell cycle.

Body

We have completed proposed research in the original Second Year Task (iii). Cell transformation will be determined by: (a) Foci Formation, (b) Anchorage-independent (soft agar) cell growth, and (c) tumor spheroid formation using new 3D HuBiogel bioassay to test whether estrogen-induced conversion of normal cells to transformed cells is inhibited by treatment with antioxidants, over expression of MnSOD, catalase, PrxIII, Trx2; or mtTFA silencing; and the part of Task (iv) that was proposed to use a novel physiologically-relevant 3D human biomatrix, HuBiogel™ assay as well as nude mice xenograft model to determine whether co treatment with STAZN, over expression of MnSOD, catalase, PrxIII, Trx2, or silencing of mtTFA in the breast cancer cells following exposure to estrogen prevents growth, angiogenesis, and invasion of tumors.

1. E2-induced ROS formation in normal breast epithelial cells: Before carrying out cell transformation, we characterized normal human mammary epithelia cells for their ability to produce ROS in response to 17 beta-estradiol (E2) exposure. These cells respond to E2 in terms of producing ROS very similar to breast cancer cells. ROS production by E2 and its metabolites, 2-OH-E2 and 4-OH-E2 in normal human mammary epithelia cells was dose-dependent (Figure 1).

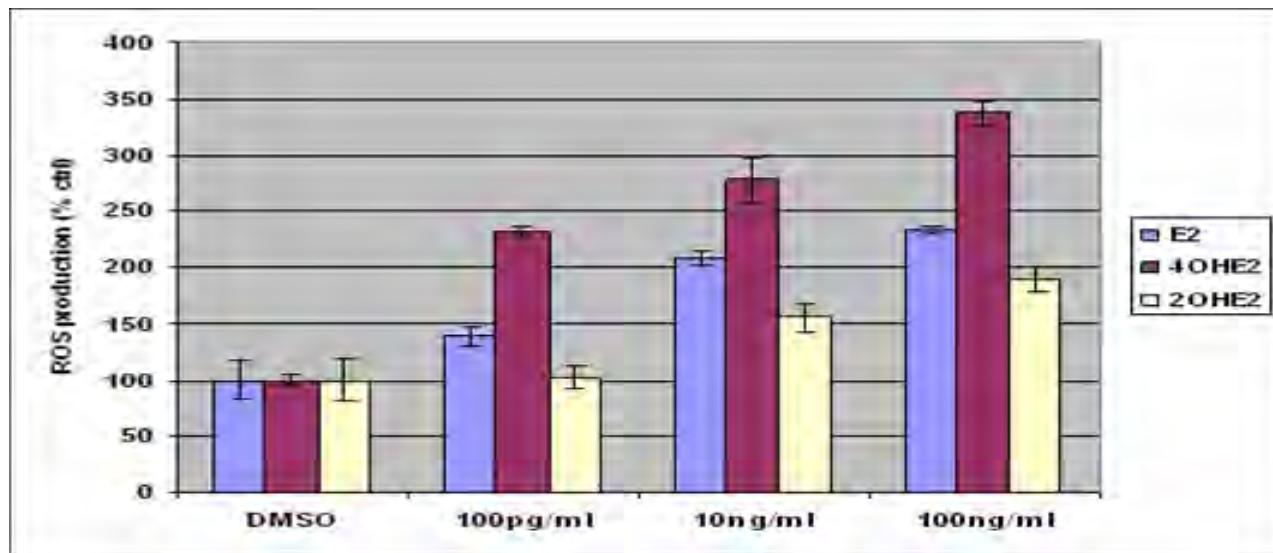


Figure 1. To determine whether estrogens and catechol metabolites induces ROS formation in normal human mammary epithelial cells, we seeded MCF-10A cells in 96well plates at 10k/well. 24hrs post seeding, medium was replaced with serum free media and cultured for 48hrs. Post starvations, DCFH-DA were diluted 1:1 in Pluronic® F-127 (20% w/v) after which it was further diluted in HBSS media at 10uM conc. DCFH-DA were

loaded into each well and incubated for 20mins. DCFDA solutions were removed and replaced with estrogens and metabolites diluted in serum free media, ROS were measured on Tecan Genios microplate reader using 485 nm and 535 nm as excitation and emission filters, respectively. Data is presented here as ROS productions with values set to controls at 100% (mean of 3-4 experiments \pm SD).

2. Dose-dependent colony formation by E2 in MCF-10A cells: We used the anchorage independent growth (AIG) assay to examine cell transforming ability of E2 by detecting AIG positive colony formation. E2 exposure to MCF-10A cells produced dose dependent increase in colony formation.

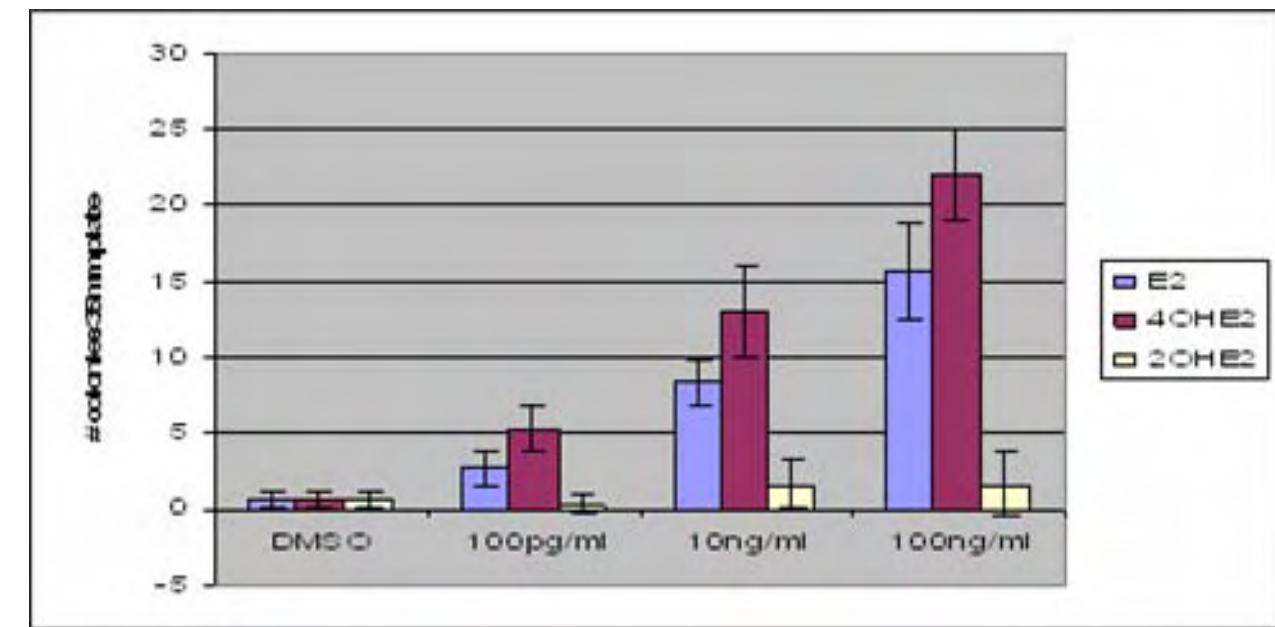
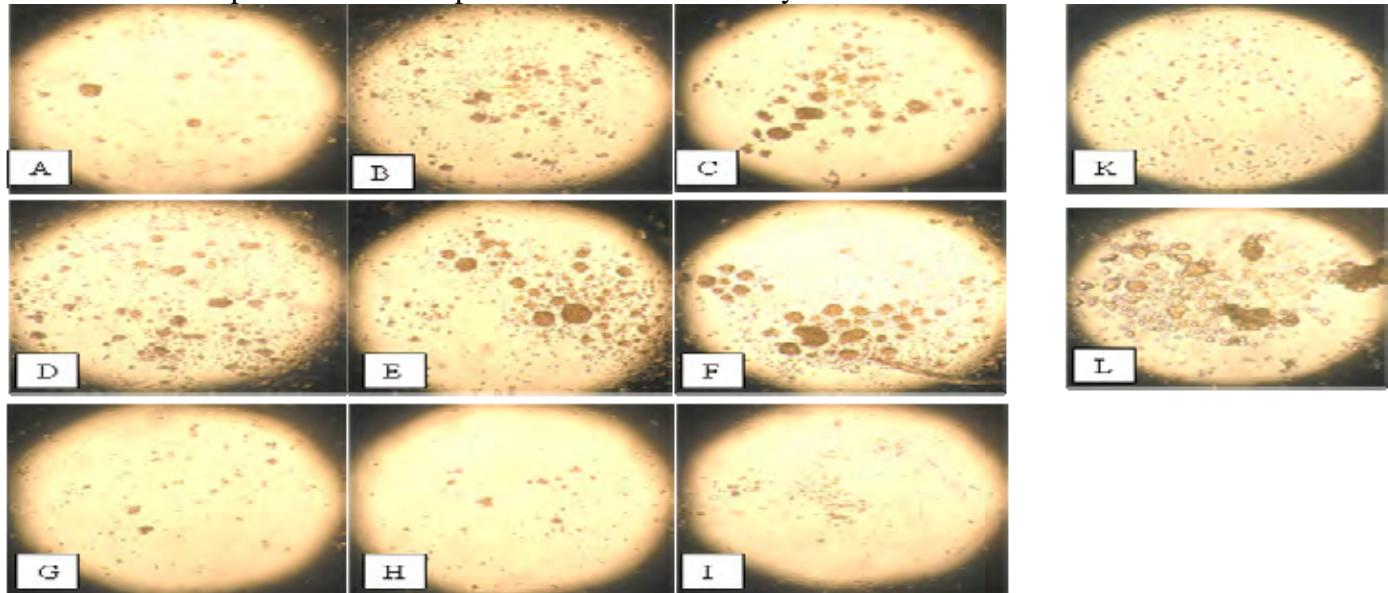


Fig 2. Representative picture of E2-induced MCF-10A transformed cells (upper panel) and the bottom panel indicates the number of colony formed with different doses of E2 and its metabolites. Cells were seeded in 6 well plates and treated with 100pg/ml, 10ng/ml and 100ng/ml dose ranges of estrogens and catechol metabolites to determine transformation abilities of these estrogens. Panel A-C are E2 treated, D-F are 4OHE2 treated and panels G-I are 2OHE2 treated. For controls, DMSO (K) and Benzo-a-pyrene (L), a potent tumorigenic compound

was used as positive control at a dose of 50 μ g/ml. Experiment were conducted four times and colonies formed in soft agar assays were enumerated as #colonies/well/24well plate (2cm^2 area) in quadruplicates wells.

3. Inhibition of E2-induced colony formation by ROS modifiers: In cells overexpressed with adenovirus construct containing catalase and MnSOD that lowers oxidant production as well as in mtTFA silenced cells, E2 produced fewer colony compared to E2 alone (Figure 2). Antioxidant ebselen also inhibited E2-induced cell transformation.

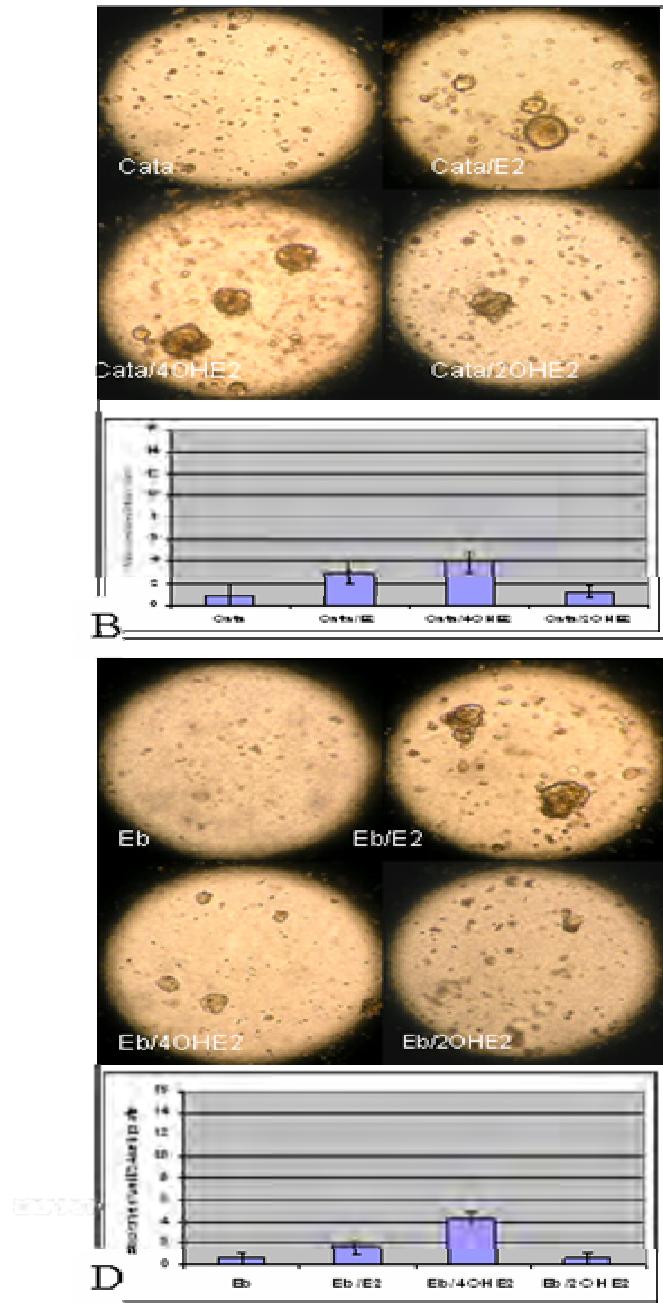
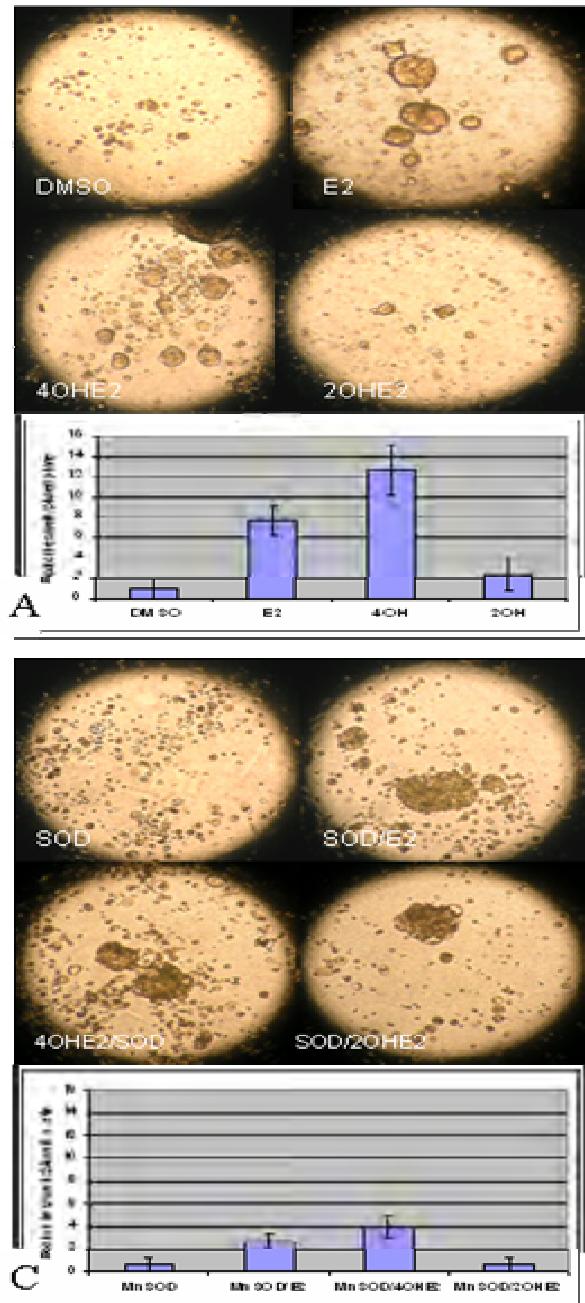


Figure 3 . To determine whether E2 induced mammary transformation is mediated by ROS, MCF-10A cells were co-treated with ROS modifiers. MCF-10A cells were infected with AdCMV-Catalase and AdCMV-MnSOD at moi 50 to overexpress these antioxidant enzymes. 48hrs post infections, chemical antioxidants were added to group and cells treated with 10ng/ml estrogenic compounds subject to transformation regimen. Colonies formed by E2 (A), is mitigated by over expression biological antioxidant catalase (B) or MnSOD (C). In addition, 40uM treatment with ebselen, a chemical antioxidant, also reduced numbers of colonies formed in mammary cells exposed to E2 or 4OHE2 (D). Experiments were conducted four times and colonies formed in soft agar assays were enumerated as #colonies/well/24well plate (2cm² area) in quadruplicates wells.

4. Clonogenic expansion of E2 transformed MCF-10A cells: To determine whether E2 induced colonies are clonogenic, we picked several colonies from each soft agar at the end of 21days and cultures them in media with 10% FBS, designated regular media (RM). Several of these clones did not passage beyond 10th passage in RM. However, of the 5 that survived up to the 21st passage, we seeded these cells at 50 k cells/well in soft agar to determine whether these cells have acquired anchorage independent growth properties, a hallmark for transformed cells and whether these clones also respond to 100pg/ml estrogens. Cells were fed twice per week and cultured for 21days. Colonies were counted from quadruplicate wells, (\pm SD). One of our clones was highly clonogenic (P21) and responsive to E2 (Figure 4).

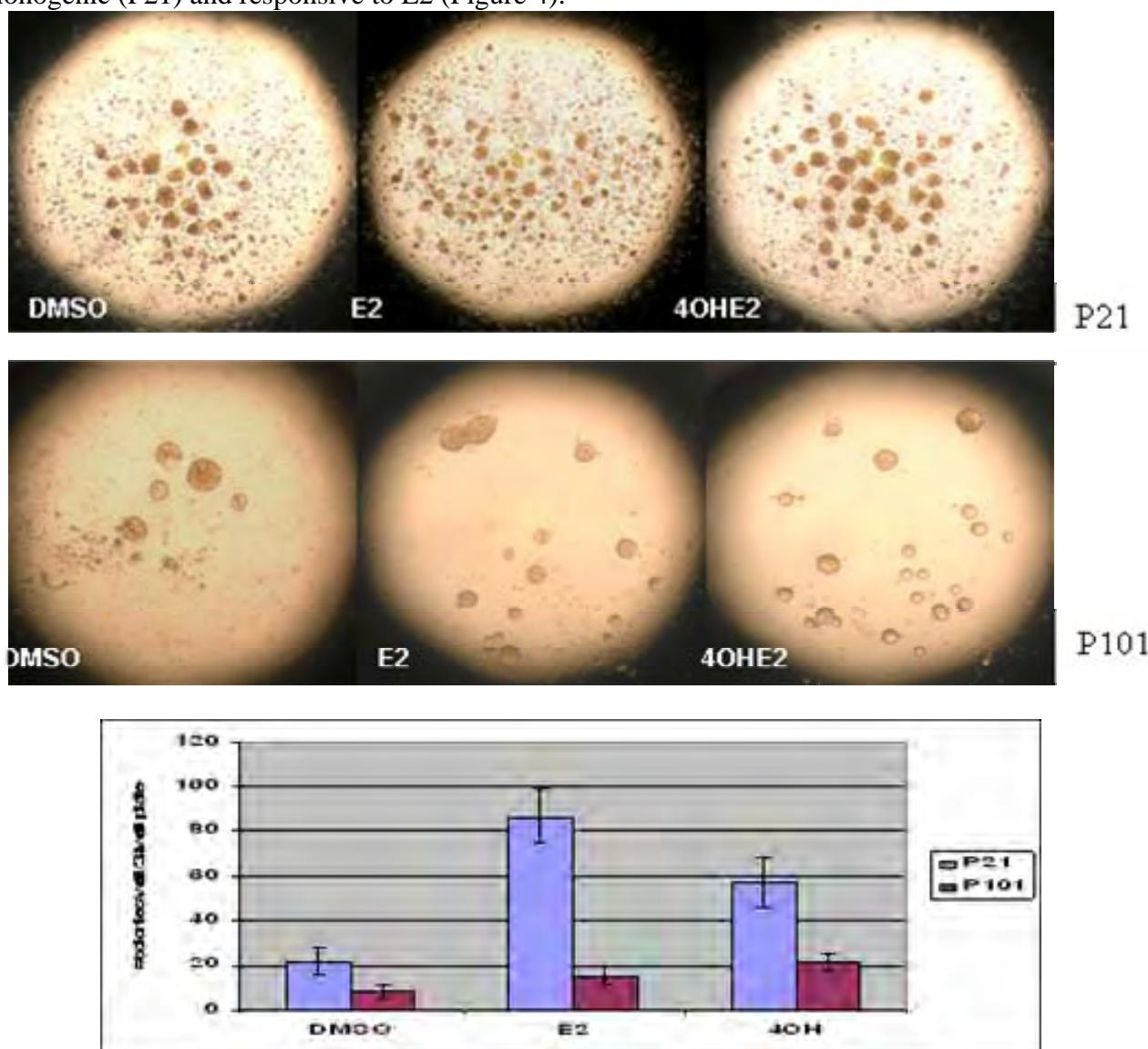
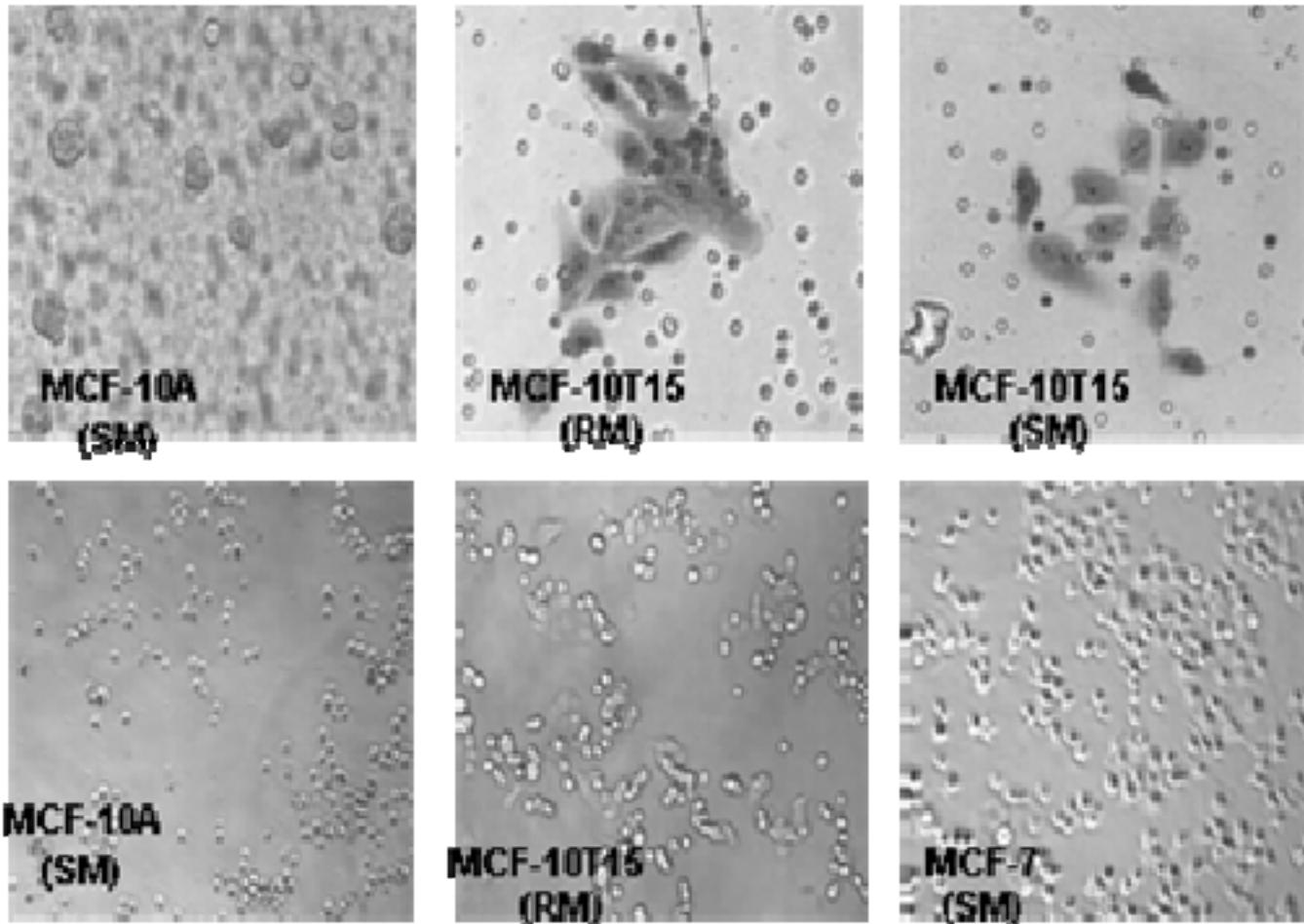


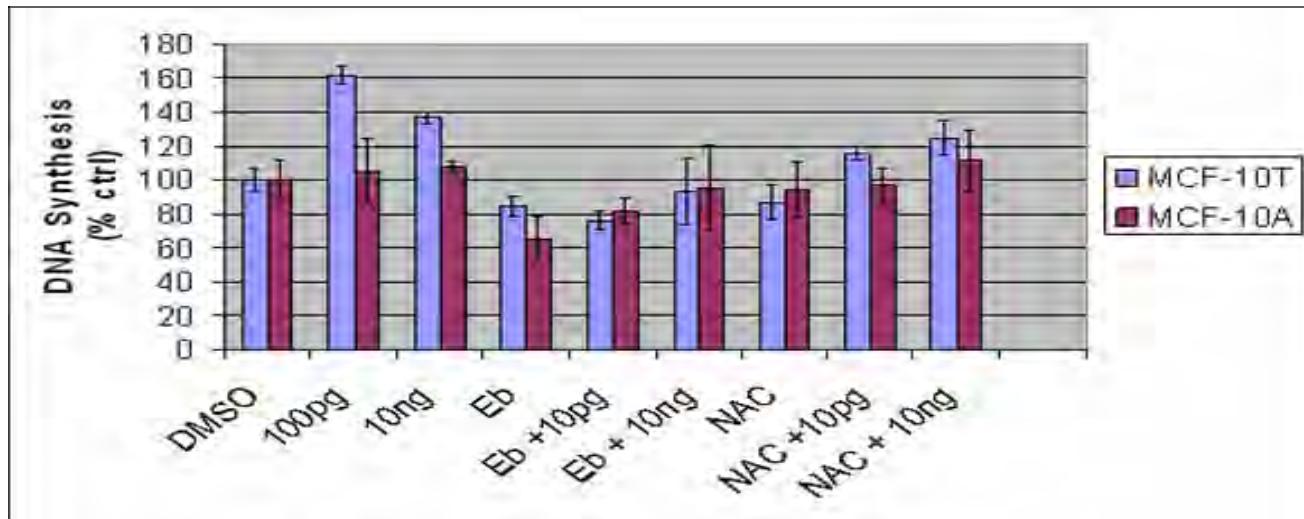
Figure 4. To determine whether growth of E2 induced transformed cells is estrogen dependent. MCF-10A transformed cells were treated with 10ng/ml estrogenic compounds subject to transformation regimen. Colonies formed by E2 were (A), is mitigated by over expression biological antioxidant catalase (B) or MnSOD (C). In addition, 40uM treatment with ebselen, a chemical antioxidant, also reduced numbers of colonies formed in mammary cells exposed to E2 or 4OHE2 (D). Experiments were conducted four times and colonies formed in soft agar assays were enumerated as #colonies/well/24well plate (2cm² area) in quadruplicates wells.

5. Analysis of invasive property of clone (MCF-10AT15) derived from E2 Induced transformation of MCF-10A: To assess invasive properties of the clones derived from E2 induced MCF-10A transformed cells, we seeded 10K MCF-10AT15 cells, a clone from MCF-10A transformation in BD BioCoatTM MatrigelTM Invasion Chambers. We also seeded these cells in glass chamber as we previously found that MCF-10A cells don't attach very well to glass in the first 16-24hrs. For the invasion assay (a), the chemottractant is either growth supplemented media (SM) or media with only 10% FBS (RM). MCF-10AT15 is highly invasive.

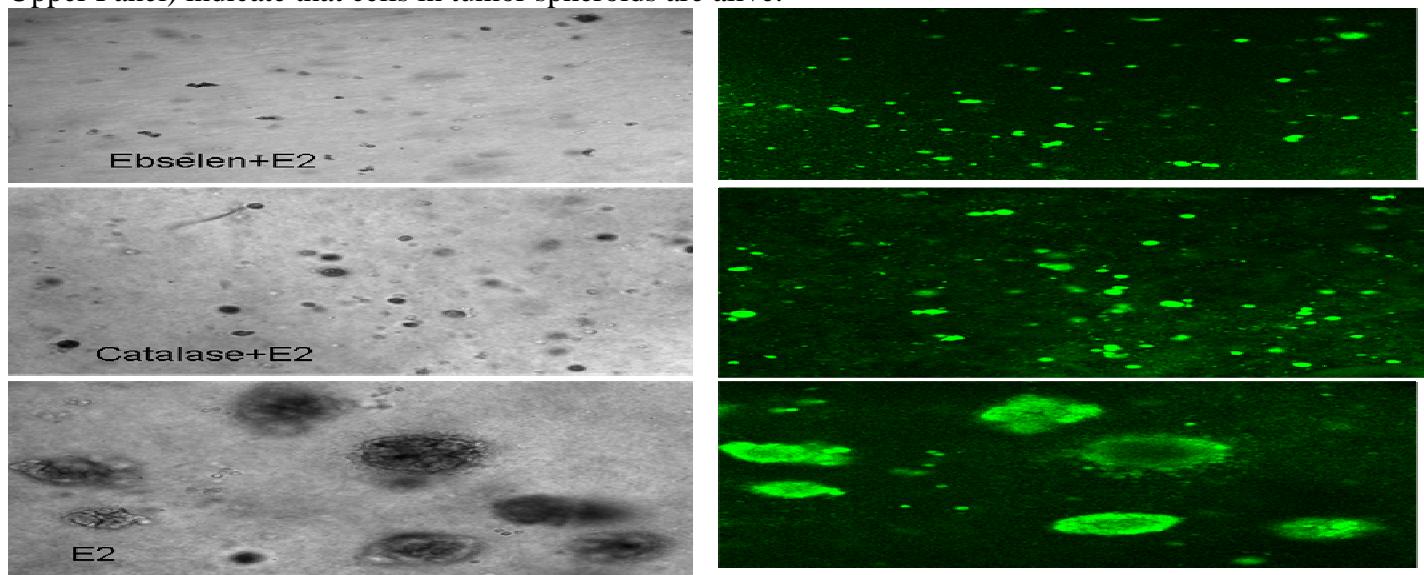


6. The growth of E2-induced transformed clone was highly responsive to E2 and was inhibited by both antioxidants, ebselen and N-acetyl cysteine. Antioxidants reduce E2-induced DNA synthesis in MCF-10A transformed cells. Cells are grown in 96-well plates for 2 days in 10% FBS DMEM/F12 and serum starved 2 days prior to addition of E2 for 18 h-48 h unless specified otherwise. Bromodeoxy uridine (BrdU) incorporation assay was used to measure DNA synthesis as indicator of apoptosis in transformed cells. Antioxidants ebselen, NAC, and catalase were pretreated for 2 h prior to the addition of E2. Colorimetric BrdUrd incorporation was measured at 450 nm with a plate reader. Results are expressed as mean OD \pm SD of three separate experiments

with control set as 100% DNA synthesis. (**) Indicates treatment significantly different from control. (*) Indicates treatment significantly different from E2. ($P < 0.05$).



7. The E2-induced 3-D tumor spheroid formation was inhibited by ROS modifiers: The tumorigenic conversion ability of E2-transformed MCF-10A cells was further investigated by 3-D culture using HuBiogel™. For 3-D culture, anchorage-independent MCF-10A human mammary gland epithelial cells transformed by E2 treatment were mixed with 3D HuBiogel™ matrix containing DMEM-F12, seeded into 55 ml rotating-wall vessels and incubated at 37°C for 16 days. These conditions allow for the spontaneous formation of tissue-like spheroids. As shown in Fig 7 below, treatment with E2 produced spheroids (Fig. 7 Left Upper Panel). Overexpression of catalase and ebselen inhibited E2-induced tumor spheroid formation. Cells were labeled with CFSE using the Vybrant kit for checking viability. All spheroids showing the green fluorophore (Fig. 7 Right Upper Panel) indicate that cells in tumor spheroids are alive.



Key Research Accomplishments: Our findings provide evidence in support of the concept that ROS functioning as signal molecules in E2-induced cell transformation. In addition to the estrogen receptor activity, E2-generated ROS may promote susceptibility to malignant transformation.

REPORTABLE OUTCOMES: Abstract submitted for Era of Hope meeting, 2008

CONCLUSIONS: We have completed proposed research in the Second Year Task (iii) and the part of Task (iv) by using: (a) Foci Formation, (b) Anchorage-independent (soft agar) cell growth, and (c) tumor spheroid formation using new 3D HuBiogel bioassay whether estrogen-induced conversion of normal cells to transformed cells is inhibited by treatment with antioxidants, over expression of MnSOD, catalase, PrxIII, Trx2; or mtTFA silencing. We previously reported that 17- β -estradiol (E2)-induced mitochondrial (mt) ROS act as signalling molecules. In the first year of this proposed study, we have shown that E2-induced cell growth was reduced by antioxidants N-acetyl-L-cysteine (NAC), catalase, and the glutathione peroxidase mimic ebselen. mtTFA siRNA transfection inhibited estrogen-induced proliferation of MCF-7 cells. Normal breast epithelial cells respond to E2 in terms of producing ROS very similar to breast cancer cells. E2 treatment to MCF-10A cells increased the formation of ROS. Over expression of catalase or silencing of mtTFA prevented E2-induced anchorage-independent growth of MCF-10A cells. We observed similar results with 3-D culture of transformed cells using HuBiogel. These results support ROS functioning as signal molecules in E2-induced cell transformation. These findings suggest that, in addition to the receptor activity of E2, *E2-generated ROS may promote susceptibility to malignant transformation*. Thus our results suggest 1) a new paradigm that estrogen-induced oxidants control cell transformation and invasiveness of transformed cells, and 2) provide the basis for the discovery of novel antioxidant-based drugs or antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer.

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APPENDICES- Copy of the ERA of HOPE 2008

Inhibition of Estrogen-Induced Growth of Breast Cancer Cells by Modulating In Situ Oxidant Levels

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The purpose of the our BCRP-funded proposal (BC051097) was to examine an untested and highly innovative concept in estrogen-induced carcinogenesis research, *i.e.*, estrogen-induced mitochondrial oxidants are involved in the promotion/progression of breast cancer through modulating signaling that controls the early G1 stage of the cell cycle. We previously reported that 17- β -estradiol (E2)-induced mitochondrial (mt) reactive oxygen species (ROS) act as signalling molecules. Here we have examined whether antioxidants, N-acetylcysteine and ebselen, overexpression of ROS lowering genes, such as, catalase and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA by real-time RT-PCR, the rate of DNA synthesis by BrD_U incorporation, and different phases of cell cycle by flow cytometry. We also determined the morphology and behaviors of cells that over-express mtSOD, catalase or silenced with mtTFA siRNA compared to those that do express these genes normally exposed to estrogen. Our data revealed that E2-induced cell growth was reduced by antioxidants N-acetyl-L-cysteine (NAC), catalase, and the glutathione peroxidase mimic ebselen. mtTFA siRNA transfection inhibited estrogen-induced proliferation of MCF-7 cells which is evident from the lower incorporation of BrD_U in SiRNA treated cells compared to wild type cells in the presence of E2. We observed similar results by flow cytometry. In E2 treated MCF7 cells, the percentage of DNA content in S phase was 18% while this decreased to 6.8% in mtTFA silenced MCF7 after 24 h. The FACS data not only confirms the results shown by the BrdUrd assay, it also shows that impairment of mitochondrial biogenesis prevents E2-induced entry of MCF7 cells into the S phase by arresting them in the G0/G1 phase. Both antioxidant treatment and detoxification of ROS prevented E2-induced expression of cyclin D1 and pcna, markers of cell proliferation detected by Real time PCR. In cells overexpressed with Adenvirus construct containing catalase that lowers oxidant production as well as in mtTFA silenced cells using their SIRNA, E2 was not able to produce any colony. Both antioxidants ebselen and N-acetylcysteine produced similar effects. It appears that E2 dependent colony formation rate of MCF-7 cells is dependent on ROS or mitochondrial signaling. Since neither antioxidants nor mitochondrial biogenesis blocker used in this study are reported to regulate the ER, our findings suggest that E2-induced mtROS modulates G₁ to S transition and some of the early G1 genes through a nongenomic, ER independent signaling pathway. These data indicate that E2-induced mtROS are involved in the regulation of early G₁ phase progression and colony formation of breast cancer cells. This work was performed under the BCRP-funded project BC051097 to DR.